Hansenula polymorpha and Saccharomyces cerevisiae Pex5p's recognize different, independent peroxisomal targeting signals in alcohol oxidase

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Hansenula polymorpha and *Saccharomyces cerevisiae* Pex5p's recognize different, independent peroxisomal targeting signals in alcohol oxidase

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Abstract Peroxisomal alcohol oxidase (AO) from *Hansenula polymorpha* is inactive and partially mislocalized to the cytosol upon synthesis in *Saccharomyces cerevisiae*.

Co-production with *H. polymorpha* pyruvate carboxylase (HpPyc1p) resulted in AO activation, but did not improve import into peroxisomes.

We show that import of AO mediated by *S. cerevisiae* Pex5p is strictly dependent on the peroxisomal targeting signal 1 (PTS1) of AO and independent of HpPyc1p.

In contrast, HpPex5p-mediated sorting of AO into *S. cerevisiae* peroxisomes is independent of the PTS1, but requires an alternative PTS that is only formed when HpPyc1p is co-produced and most likely involves folding and co-factor binding to AO.

Keywords: Peroxisome; Protein translocation; Pex5p; Enzyme activation; Yeast

1. Introduction

Most peroxisomal matrix proteins contain a peroxisomal targeting signal 1 (PTS1) that consists of a tripeptide located at the extreme C-terminus of the protein. The PTS1-receptor, Pex5p, recognizes this signal via its C-terminal TPR (tetratricopeptide repeats) domain. The N-terminal half of Pex5p's is important for binding to other components of the peroxisomal protein translocation machinery.

Peroxisomal alcohol oxidase (AO) is a key enzyme of methanol metabolism in methylotrophic yeast (e.g., *Hansenula polymorpha* and *Pichia pastoris*). AO follows a remarkable sorting mechanism. It contains a PTS1 (–ARF) that is sufficient to direct a reporter protein to peroxisomes [1,2], but is redundant for AO sorting. Nevertheless, AO import depends on Pex5p, but its C-terminal TPR domain is not required [3]. Surprisingly, the cytosolic enzyme pyruvate carboxylase (Pyc1p) is essential for AO sorting and activation, most likely by being involved in FAD-binding to AO monomers [4].

Attempts to introduce enzymatically active AO in peroxisomes of bakers yeast failed so far [5,6]. Reconstitution of the AO sorting and assembly pathway in this heterologous host may add to the understanding of this process.

Here, we show that co-production of *H. polymorpha* pyruvate carboxylase (HpPyc1p) results in activation of AO in bakers yeast. Analysis of AO sorting in *Saccharomyces cerevisiae* revealed that HpPex5p and ScPex5p recognized different, independent peroxisomal targeting signals in AO.

2. Materials and methods

2.1. Organisms and growth

Hansenula polymorpha NCYC 495 (*leu2*) [7], *S. cerevisiae* CEN.PK2-1D (MATα, *leu2[3,112]*; *ura3-52; his3*-Δ1; *MAL2-8^C*; *SUC2*) [8] and CEN.PK700 (MATa, *leu2[3,11]*; *ura3-52his3*-Δ1; *MAL2-8^C*; *SUC2*; *pyc1[41,3501]::loxP-Kan-loxP*; *pyc2[41,3496]::loxP-Kan-loxP*), in which *PYC1* and *PYC2* were deleted, were used. All *S. cerevisiae* strains were derived from these strains, except for UTL7A *pex5* [9] (see Table 1). *S. cerevisiae* cells were grown in 0.67% yeast nitrogen base (Difco, Sparks, ME) containing 1% glucose. When needed, uracil (30 mg/L), leucine (30 mg/L) or histidine (20 mg/L) was added. *Escherichia coli* DH5α was used for cloning purposes and cultivated as described [10].

2.2. Construction of plasmids and strains

An *H. polymorpha* pyruvate carboxylase (Hp*PYC1*) expression plasmid was constructed as follows. The 0.6 kb *Bam*HI fragment of plasmid pBKK7, containing the *MET25* promoter (P_{MET25}), was cloned into *Bam*HI-digested pRS416. The Hp*PYC1* gene was cloned into *Sma*I-digested pRS416-P_{MET25} as *Nco*I/*Nhe*I (Klenow-filled in) fragment of the pHIPX5-PYC1 plasmid [4]. The obtained plasmid of 9.9 kb was designated pP_{MET25}HpPYC1. The *URA3* gene in this plasmid (*Swa*I–*Eco*RI fragment of 7293-bp Klenow-filled in) was replaced by a 2134-bp *Hpa*I fragment of YEp13 containing the *LEU2* gene, resulting in plasmid pP_{MET25}HpPYC1-LEU2. This plasmid, linearised by *Eco*RI, was used to transform *Sc pyc1*, *pyc2* strain [11].

Leucine prototrophic transformants were selected. Integration in the *leu2* locus was confirmed by Southern blotting.

The AO gene (*AOX*) was cloned into YEplac195 [12] behind the triosephosphate isomerase promoter (P_{TPI1}) as follows. The *AOX* gene was amplified using primers "AOX ATG" containing a *Spe*I-recognition site and "AOX stop", carrying a *Cla*I-recognition site (Table 2). To facilitate cloning, the amplified gene was introduced into *Sma*I-digested pBluescript® II KS(+) (Stratagene, La Jolla, CA). The correct sequence was confirmed by sequencing. A 2036-bp *Spe*I–*Pst*I fragment of pBS-AOX containing *AOX* was introduced downstream of P_{TPI} in plasmid YEplac181-P_{TPI1}-T_{CYC1} digested with *Xba*I–*Pst*I. The 3533-bp expression cassette containing promoter, gene and terminator was isolated using *Pvu*II and inserted to YEplac195 digested with the same enzyme. The resulting 8452-bp plasmid was designated YEplac195-P_{TPI1}AOX-T_{CYC1} and was used to transform *S. cerevisiae* WT and HpPYC1 strains.

Plasmid YEplac195-P_{TPI1}AOX-LARA-T_{CYC1}, encoding AO in which the C-terminal phenylalanine was replaced by alanine (AO^{mut}) was made by replacement of the *Pst*I (T4 DNA polymerase-blunt ended)/*Sal*I *AOX*-fragment in YEplac195-P_{TPI1}AOX-T_{CYC1} with a 1105-bp *Sal*I/*Stu*I fragment of pHIPX1-AOX-LARA [3].

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Abbreviations: AO, alcohol oxidase; GFP, green fluorescent protein; Pyc, pyruvate carboxylase; PTS, peroxisomal targeting signal

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The *S. cerevisiae* strains HpPEX5 and HpPYC1-HpPEX5, in which Sc *PEX5* was replaced by Hp*PEX5* under control of the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GPD}), were constructed using PCR-directed integration [13]. The *S. cerevisiae HIS3* gene was amplified using primers "HIS start" and "HIS stop" (Table 2) and inserted into *Sma*I-digested pBluescript®II KS(+), resulting in pBS-HIS3. The Hp*PEX5* gene was introduced downstream from P_{GPD} in *Sma*I-digested p426GPD [14] as a *Hin*dII/*Hin*dIII (Klenow-filled in) fragment of pHIPX4-PEX5 [15]. The *Sac*I/*Asp718*I (T4 DNA polymerase-blunt ended) fragment of p426GPD-HpPEX5, containing the expression cassette with P_{GPD}, Hp*PEX5* and the *CYC1* terminator (T_{CYC1}) was recloned in pBS-HIS3 digested with *Sac*I/*Dra*III (T4 DNA polymerase blunt-ended). This plasmid, pBS-HIS3-P_{GPD}-HpPEX5, was used as template to amplify the Hp*PEX5* expression cassette containing P_{GPD}-Hp*PEX5*-T_{CYC1}-Sc *HIS3*. To this end, primers "P_{GPD}-HpPEX5 start" and "P_{GPD}-HpPEX5 stop" (Table 2) were used that were extended with 50 nucleotides at the 5′ terminus representing sequences upstream and downstream of Sc *PEX5[13]*. The obtained fragment of 4730 bp was used to transform *S. cerevisiae* WT and HpPYC1 strains. Histidine prototrophic transformants were selected and replacement of Sc *PEX5* was confirmed by PCR using primers "HpPEX5 colony PCR 1" and "HpPEX5 colony PCR 2" (Table 2).

For production of green fluorescent protein (GFP)–SKL in *S. cerevisiae* WT and HpPEX5 strains, cells were transformed with pGFP-SKL [16].

2.3. Miscellaneous

Electron microscopy, immunocytochemistry, fluorescence microscopy [17], AO activity measurements and the separation of AO monomers and octamers [18] were performed as described. SDS–PAGE and Western blotting were performed by established techniques.

3. Results

3.1. H. polymorpha *AO is activated in* S. cerevisiae *expressing HpPYC1*

To test whether HpPyc1p activates AO in *S. cerevisiae*, a strain was constructed that contained the *H. polymorpha AOX* and *PYC1* genes, but lacked Sc*PYC1* and Sc*PYC2* (strain HpPYC1-AO, see Table 1). In these cells HpPyc1p levels were obtained that were comparable to those present in methanol-grown *H. polymorpha* WT cells, however the AO protein levels were much lower in HpPYC1-AO relative to *H. polymorpha* (*Hp*; Fig. 1A).

AO enzyme activities of 0.2 (±0.02) U/mg protein were measured in cell extracts of HpPYC1-AO. As expected AO activity was not detectable in the control *S. cerevisiae* strain that produces AO, but lacks HpPyc1p (WT-AO). These data indicate that co-expression of Hp*PYC1* with Hp*AOX* is sufficient to obtain enzymatically active AO in *S. cerevisiae*. Like the AO protein levels also the enzyme activities were significantly lower in *S. cerevisiae* HpPYC1-AO compared to methanol-grown *H. polymorpha* WT controls (*Hp*, Fig. 1A [4]).

Sucrose density centrifugation of cell free extracts confirmed that in the presence of HpPyc1p AO octamers were formed in *S. cerevisiae*, whereas in cells lacking HpPyc1p AO remained monomeric (Fig. 1B). Upon cytochemical staining of AO enzyme activity, AO activity was only detected inside peroxisomes in cells producing both AO and HpPyc1p (data not shown).

Table 1
S. cerevisiae strains used in this study

Strain	*S. cerevisiae* gene		*H. polymorpha* gene				Source
	PYC1/PYC2	*PEX5*	*PYC1*	*PEX5*	*AOX*	*AOX (LARA)*	
Pex5 (UTL7A *pex5*)	+	–	–	–	–	–	[9]
WT (CEN.PK2)	+	+	–	–	–	–	[8] Dr. Peter Koetter
pyc1, *pyc2* (CEN.PK700)	–	+	–	–	–	–	Dr. Peter Koetter
HpPYC1	–	+	+	–	–	–	This study
HpPYC1–AO	–	+	+	–	+	–	This study
HpPYC1-AO^{mut}	–	+	+	–	–	+	This study
HpPYC1-HpPEX5	–	–	+	+	–	–	This study
HpPYC1-HpPEX5-AO	–	–	+	+	+	–	This study
HpPYC1-HpPEX5-AO^{mut}	–	–	+	+	–	+	This study
WT-AO	+	+	–	–	+	–	This study
WT-AO^{mut}	+	+	–	–	–	+	This study
HpPEX5	+	–	–	+	–	–	This study
HpPEX5-AO	+	–	–	+	+	–	This study
HpPEX5-AO^{mut}	+	–	–	+	–	+	This study
WT-GFP-SKL	+	+	–	–	–	–	This study
HpPEX5-GFP-SKL	+	–	–	+	–	–	This study

Table 2
Oligonucleotide primers used in this study

Primer name	Sequence 5′–3′
AOX ATG	GGACTAGTCAAAATGGCCATTCCTGACG[a]
AOX stop	CCATCGATGTCCTTCCACGTCTCC[a]
HIS start	GCCGAATTCCCTAGCATGTACGTGAG
HIS stop	GGTAGGCGCCACCTATCACCACAAC
P_{GPD}-HpPEX5 start	TTAGTTCCTATTTTTGGATATATATACATCAATAAACAATA TATCATAACACTAGGGTAGGCGCCACCTATCACCACAAC
P_{GPD}-HpPEX5 stop	CTAATGAATTTGGGCAGTGATGCGAGAACATAAAATTGCG GAGAACCATAAACCGTCTATCAGGGCGATGGCCCACC
HpPEX5 colony PCR 1	TATGGATCAGCGCAAGGTCG
HpPEX5 colony PCR 2	GTGGTGTAGTACTGCATCTC

[a]Underlined nucleotides indicate restriction sites.

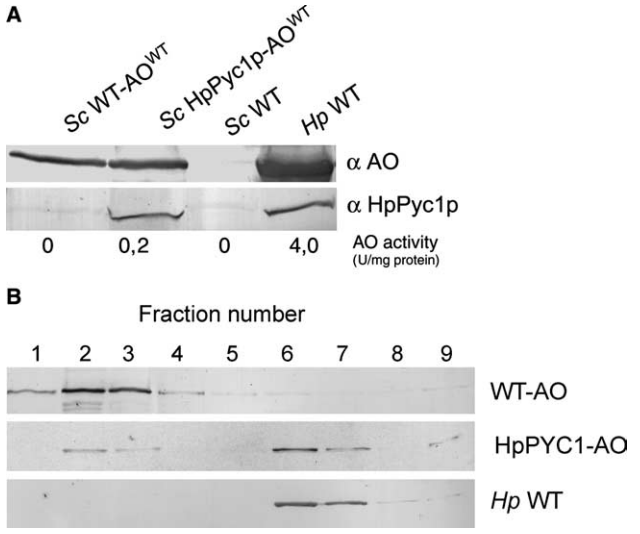

Fig. 1. (A) AO and HpPyc1 protein levels in glucose-grown *S. cerevisiae* cells and methanol-grown *H. polymorpha* wild type controls (*Hp*WT). Equal amounts of protein were loaded per lane. Blots were decorated with the indicated antibodies. AO enzyme activities are expressed as U/mg protein. (B) Sucrose density centrifugation for separation of AO monomers and octamers [18]. Intact cells, cell debris and protein aggregates were removed from the cell extracts by a clarifying spin at $30000 \times g$ prior to loading onto sucrose gradients. Fractions were analysed by Western blotting using α-AO antibodies. AO octamers present in *Hp*WT and HpPYC1-AO sediments to fractions 6 and 7, whereas AO monomers that accumulate in WT-AO sediment to fractions 2 and 3. Equal portions of each fraction were loaded per lane.

3.2. *Co-expression of* H. polymorpha *PYC1 or PEX5 does not improve the efficiency of AO sorting in* S. cerevisiae

AO localization studies using WT-AO cells (Fig. 2) revealed that only a minor portion of the AO protein was present in peroxisomes, whereas the bulk was mislocalized to the cytosol and nucleus, where it often was present in protein aggregates. This dual localization was unchanged in HpPYC1-AO cells (data not shown), which indicates that co-expression of Hp*PYC1* does not significantly improve AO import into *S. cerevisiae* peroxisomes.

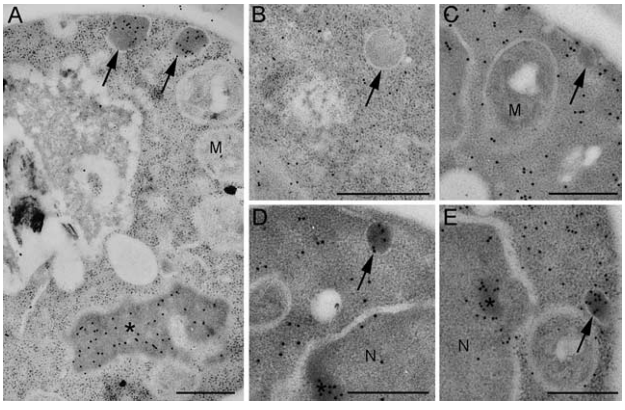

Fig. 2. Immunolabeling experiments demonstrating the subcellular localization of AO. In WT-AO cells AO is partially imported to peroxisomes (arrow; A), but not in WT-AO^{mut} (B) or HpPEX5-AO cells (C). AO is also partially sorted to peroxisomes of HpPYC1-HpPEX5-AO (D) and HpPYC1-HpPEX5-AO^{mut} (E) cells. The bar represents 0.5 μm. AO aggregates are indicated by an asterisk. M, mitochondrion; N, nucleus.

Recently, we showed that AO contains an alternative, yet unknown PTS, that most likely is formed upon HpPyc1p-mediated FAD binding and recognized by the N-terminal domain of HpPex5p [3]. We next tested whether replacement of Sc*PEX5* by Hp*PEX5* improved AO import. In a strain producing both HpPyc1p and HpPex5p (HpPYC1-HpPEX5-AO) only a minor portion of AO was imported into peroxisomes (Fig. 2D). This low import efficiency was not due to limiting HpPex5p levels, because these were similar as in *H. polymorpha* WT controls (Fig. 3A).

3.3. *HpPex5p and ScPex5p recognize different peroxisomal targeting signals in AO*

In a strain in which Sc*PEX5* was replaced by Hp*PEX5* without co-expression of Hp*PYC1*, anti-AO specific labelling was absent in peroxisomes (Fig. 2C), which confirms our earlier data that HpPex5p does not bind AO via its PTS1 [4]. However, ScPex5p most likely imports AO via its PTS1, because AO is partially imported into peroxisomes of WT-AO cells (Fig. 2A). To test this, the PTS1 (–ARF) of AO was destroyed by changing it into –ARA (AO^{mut}) [3]. In cells of this strain (WT-AO^{mut}) sorting of AO to peroxisomes was fully abolished (Fig. 2B). However, AO^{mut} was imported into peroxisomes in cells producing both HpPyc1p and HpPex5p (HpPYC1-HpPEX5-AO^{mut}; Fig. 2E).

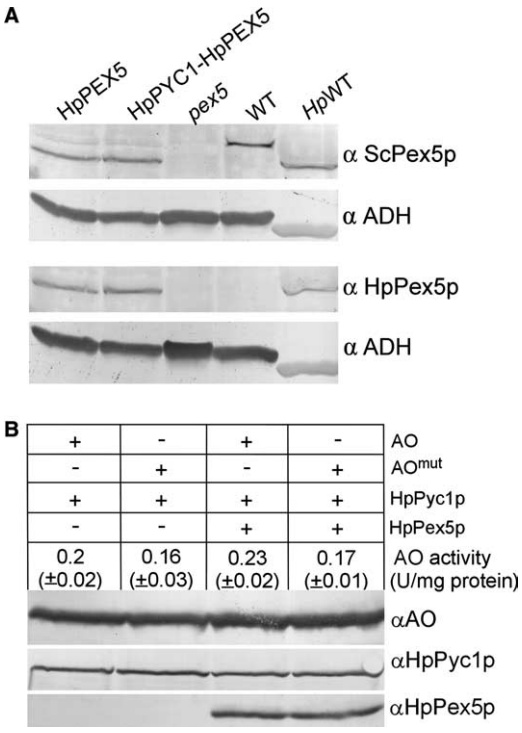

Fig. 3. (A) HpPex5p levels in *S. cerevisiae* strains are similar as observed in *H. polymorpha* WT controls (HpWT). Western blots were decorated with the indicated antibodies. Anti-ScPex5p antibodies cross-react with HpPex5p. ScPex5p has a slightly higher apparent molecular weight compared to HpPex5p (upper blot). The anti-alcohol dehydrogenase (ADH) antibodies are raised against ScADH, but also recognize HpADH, which has a slightly lower apparent molecular weight. Equal amounts of protein were loaded per lane. ADH was used as a loading control. (B) Cells producing the indicated proteins were grown in glucose-containing media and analysed for AO protein and enzyme levels. Equal amounts of protein were loaded per lane. Blots were decorated with the indicated antibodies. Enzyme activities (+ standard deviations) are expressed as U/mg protein.

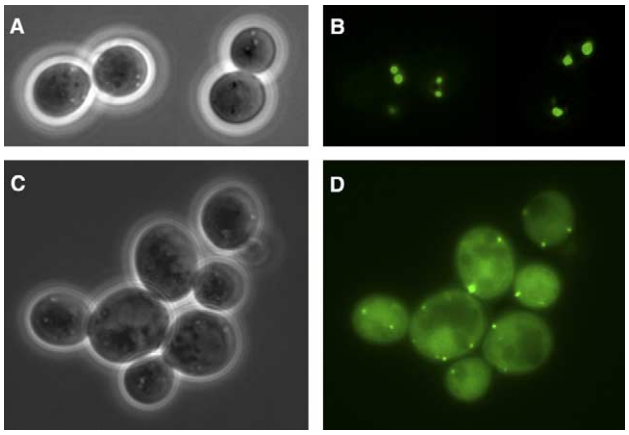

Fig. 4. Fluorescence microscopy of WT-GFP-SKL (A,B) and HpPEX5-GFP-SKL cells (C,D). A,C – bright field; B,D – fluorescence images.

Hence, in *S. cerevisiae* the alternative AO PTS is formed in an HpPyc1p-dependent way and recognized by HpPex5p. However, because no AO import was observed in HpPYC1-AO^{mut} cells (data not shown), we conclude that ScPex5p is unable to recognize this alternative PTS.

All *S. cerevisiae* strains that produced AO in the presence HpPyc1p showed similar AO enzyme and protein levels, independent of the localization of the protein. This indicates that activation of AO in *S. cerevisiae* is not dependent on import of the protein into the peroxisomal matrix (Fig. 3B).

3.4. *HpPex5p is not fully functional in PTS1 protein import in* S. cerevisiae

Partial import of AO in strains producing both HpPex5p and HpPyc1p suggests that HpPex5p is not fully functional in the heterologous host. We therefore analysed import of green fluorescent protein containing a PTS1 in cells expressing Hp*PEX5* (HpPEX5-GFP-SKL). As shown in Fig. 4, most GFP fluorescence was mislocalized to the cytosol, whereas only part of the fluorescence was found in punctuated structures. In contrast, GFP-fluorescence was confined to punctuated structures in WT controls. The growth rate and final yield of HpPEX5-GFP-SKL cells on oleic acid media was reduced as compared to WT controls (data not shown), which is also indicative for a partial matrix protein import defect.

4. Discussion

Here, we show that pyruvate carboxylase of *H. polymorpha* (HpPyc1p) is sufficient to activate AO in the heterologous host *S. cerevisiae*. We previously cloned Hp*PYC1* by complementation of a *H. polymorpha* mutant defective in AO assembly [4,19]. This finding was unexpected since HpPyc1p is an enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate. Mutational analysis revealed that not the enzyme activity, but another function of this protein fulfils a role in AO activation. Most likely HpPyc1p mediates FAD-binding via a yet unknown mechanism.

S. cerevisiae contains two pyruvate carboxylase genes, *PYC1* and *PYC2*. Although the corresponding proteins are approximately 75% identical to HpPyc1p, they are unable to activate AO.

Our data suggest that HpPyc1p is the only *H. polymorpha* protein that is specifically required for AO activation. All *S. cerevisiae* strains that produced AO in the presence HpPyc1p showed similar AO activity levels indicating that activation is not dependent on import into peroxisomes.

Our data reveal that ScPex5p binds the PTS1 of AO (–ARF). This PTS1 is a conserved variant of the classical PTS1, –SKL, which most likely has a relatively low affinity for ScPex5p [20,21]. This may explain the low efficiency of ScPex5p mediated AO import. Replacement of Sc*PEX5* by Hp*PEX5* did not improve import, because HpPex5p is not fully functional in *S. cerevisiae* (Fig. 4). Other examples have been reported where the *PEX5* gene from one organism fails to fully complement *PEX5* deletion in another organism (e.g., *Pichia pastoris PEX5* and *Penicillium chrysogenum PEX5* do not fully restore the PTS1 import defect in *H. polymorpha pex5* cells [15,22]).

HpPex5p binds a yet unknown, alternative PTS in AO, which is not recognized by ScPex5p. On the other hand, HpPex5p apparently is unable to recognize the AO PTS1 (-ARF) in the context of the AO protein [3]. However, fusion of this PTS1 to GFP (GFP-ARF) results in import of this reporter protein into *H. polymorpha* peroxisomes [2]. A likely explanation is that residues that lie upstream from the C-terminal –ARF interfere with AO-binding to the PTS1-binding TPR domain of HpPex5p, but not of ScPex5p.

Because ScPex5p-mediated AO targeting does not depend on HpPyc1p, it is possible that both FAD-containing and FAD-lacking AO is imported into peroxisomes by ScPex5p. The special AO PTS probably prevents import of FAD-lacking AO in *H. polymorpha*. Our results imply that HpPex5p is capable to sense binding of FAD to the cargo protein. *S. cerevisiae* may have developed a similar mechanism for import of the peroxisomal flavo-enzyme acyl CoA-oxidase, which is also imported via the N-terminal domain of ScPex5p [16,23]. The involved functional domains in the N-terminus of HpPex5p and ScPex5p are not the same, because ScPex5p does not recognize the alternative PTS of AO, whereas the region in ScPex5p that is essential for binding of acyl CoA oxidase [23] is absent in HpPex5p.

It is possible that co-factor binding in the cytosol is a very common feature in peroxisomal protein translocation, like in the Tat protein translocation pathway. For instance, in *Y. lipolytica* proper assembly of the heteropentameric acyl-CoA oxidase is essential to allow import into peroxisomes [24]. The importance of co-factor binding and folding in peroxisomal protein import may so far have been overlooked due to the common use of non-peroxisomal reporter proteins in peroxisomal protein translocation studies.

If the N-terminal domains of Pex5p's can sense co-factor binding, it is tempting to speculate that in PTS2 protein import the Pex7p accessory proteins (Pex18p, Pex21p, Pex20p and the long form of human Pex5p) fulfil a similar role.

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